GM REF NO: GM -

UNIVERSITY OF ABERDEEN

Application to Foresterhill Genetic Modification Safety Committee for approval to carry out work with genetically modified micro-organisms (GMMs)

Important

Obtain a copy of the SACGM's Compendium of Guidance before completing this application. The Compendium provides guidance on risk assessment of GMMs and the containment measures required. Copies of the Compendium can be borrowed from The School Office, School of Medical Sciences, IMS. The Compendium is also at http://www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/index.htm. Further guidance on genetic modification can be found at http://www.hse.gov.uk/biosafety/gmo/information.htm

Note: The definition of 'genetic modification' means that there is a permanent change to an organism's genetic material which can be inherited and that the method used to achieve the change is artificial, and not based on the natural processes of mating, selection or recombination. Approval is required for all work involving GMMs, including stably transfected mammalian cell lines, even if others have supplied the modified organisms and there is no intention to genetically modify them further.

- Your responses to the sections should not be limited by the sizes of the boxes on this form. Expand the table in the electronic version of form as necessary to accommodate your responses.
- Applications will be considered by a committee composed of both specialists and non-specialists in genetic modification.
  Your application should be comprehensible to non-specialist scientists.

1.	**Title of project**	**Genetic manipulation of *E.coli* by the 2014 University of Aberdeen iGEM (International Genetically Engineered Machine Competition) team**
2.	**Proposer (must be Project Leader)** This will normally be the most senior member of staff in the group who has involvement in, and responsibility for, the project.	Berndt Müller/Ian Stansfield
3.	**School**	Medical Sciences
4.	**Building**	Polwarth Building/IMS
5.	**Laboratory** *Give details of all laboratories which will be used*	Polwarth 2:054 (Teaching Labs)
6.	**List other facilities which will be used and confirm that those in charge of the facilities are aware of this application** *For example, biological service units, plant growth units, specialist equipment suites*	FACS facility
7.	**Names of those other than the proposer, who will have a supervisory role on the project**	Dr Suzi Black Dr Rey Carabeo Dr Stefania Spano The Project will also be supported by staff technicians Alison Davidson Kelly Reid
8.	**Previous experience of key individuals in genetic modification**	Berndt Müller, Rey Carabeo and Ian Stansfield have each more than 20 years of experience of working with microrganisms and in genetic modification, Stefania Spano and Suzi Black have 19 and 10 years of this experience, respectively.

9. Overview of the project

Include
(a) scientific goals,
(b) details of recipient micro-organism (including strain number of micro-organisms),
(c) details of vectors,
(d) details of genes being modified,
(e) an estimation of culture volumes which will be used

a) Establish proof-of principle for an *E. coli* surface display strategy for detecting disease markers.

The project will engineer *E. coli* to produce a system for the detection of the presence of two molecules, for example two disease proteins ("antigens"), on a cell surface (see Figure 1). Two *E. coli* strains will be produced that express on the cell surface proteins that are able to bind with high specificity to one or the other of these molecules ("Ag43 antibody fusions"). The strains will be marked by plasmids expressing different fluorescent proteins ("CYFP" and "RFP"). In addition they will be engineered to contain a quorum sensing system to ensure cell-cell communication. One of the strains will contain a quorum sender plasmid expressing the LuxI enzyme generating the N-acyl homoserine lactone signalling molecule (3-oxohexanoyl-homoserine lactone; abbreviated AHL). The other strain will contain a quorum sensor plasmid responsive to AHL and containing a green flourescent protein (GFP) gene whose expression is upregulated by AHL.

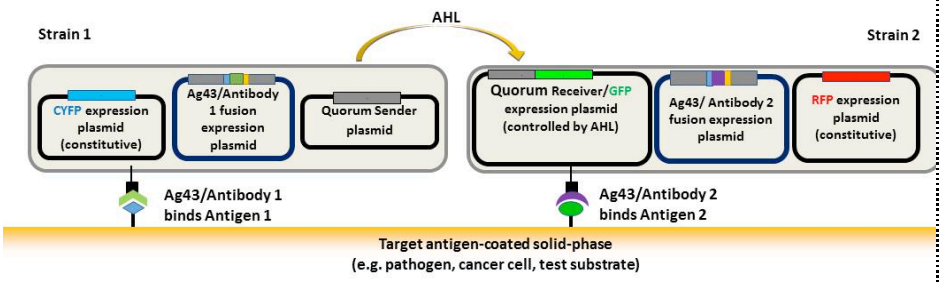

Figure 1. *E. coli* surface display strategy for detecting disease markers

Binding of the two *E. coli* strains to a surface containing both molecules will lead to an increased local concentration of both strains. Cell-cell communication mediated by the quorum sensing sender and sensor plasmids will result in increase of AHL levels and in GFP expression, allowing the detection of the presence of both molecules using approaches such as fluorescent microscopy or fluorescent activated cell sorting that can detect and measure GFP expression.

To establish proof-of-principle we will produce a plasmid for the targeting of protein sequences (epitope tag or antibody fragment) to the *E. coli* outer cell wall. This will be achieved by creating a plasmid expressing the outer cell wall component Antigen 43 (Ag43) protein, and modifying this plasmid by including a cassette in the Ag 43 open reading frame with extra coding sequences encoding epitope tags or antibody fragments. In first experiments we will express epitope tags (FLAG tag or similar) that allow the capturing of *E. coli* on a solid surface such as for example sepharose beads coated with anti-epitope antibodies. In the future (most likely after the completion of this project) this can be replaced with antibody fragments. These plasmids are key parts of this project and will be deposited in the iGEM registry of parts.

Alternative approaches/backup-plans.
In case surface presentation using Ag43 fails we will present proteins at the surface using the *Pseudomonas syringae* Ice Nucleation Protein (INP). INP is localised in the outer membrane surface and has been

previously used for the presentation of protein domains such as for example antibodies on the surface of *E. coli* in the iGEM competition (http://2012.igem.org/Team:Penn/SurfaceDisplayOverview).

In case quorum sensing is inefficient we will enhance cell-cell interactions by expressing streptavidin fused to INP in one *E. coli* strain and the The Biotinylation Acceptor Peptide motif fused to either AG43 or INP in a second strain. The Biotinylation Acceptor Peptide motif is biotinylated by the biotin ligase BirA and will also be exposed at the cell surface as an Ag43 or INP fusion protein. Cell-cell interactions will be promoted by the interaction between biotin and streptavidin exposed at the cell surface.

Ultimately this system may be developed for the detection of any pair of biomolecules that bind specifically to protein molecules such as antibodies.

b) Details of microorganisms

Strains of *E.coli* to be used (DH5α, Top10 and possibly other closely related strains) are all derivatives of the disabled K12 strain. The exception is BL21, which may be used if high-level expression of some genes is needed (see below). BL21 [DE3] is considered inherently safe, in the sense that it is broadly equivalent to K12, and offers no additional risks to the use of K12 derivatives (Chart et al., (2000) J.Applied Microbiol. 89, 1048-1058). It is believed to be unlikely to colonise and establish a persistent infection in the gut of a healthy individual. Overall, *E.*coli is considered inherently safe.

Strain details:
DH5α: (F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1) or a similar cloning strain.
Top10: (F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-).
K12 derivatives with single gene knockouts (delta BirA).
BL21(DE3): *E. coli* B F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])

c) Details of vectors.

Vectors used in this project are either 1) standard cloning vectors or 2) vectors designed for and obtained through the iGEM Registry of Standard Biological Parts.

1) Standard cloning vectors include pGEM derivatives, pET vectors, and pBR322 derivatives.

2) Vectors designed for, and used in, the iGEM competition. These vectors are listed at the following web site: http://parts.igem.org/Plasmid_backbone

This site, and the link through to the Registry of Standard Biological Parts, lists the complete catalogue of standard engineering biology parts. Some of these Parts (vectors) have been chosen to be used in this project and are listed in the attached parts list. The vector backbones of these parts are:

pSB1A2; a high copy number plasmid carrying ampicillin resistance. The replication origin is a pUC19-derived pMB1 (copy number of 100-300 per cell). (http://parts.igem.org/Part:pSB1A2)

pSB1AK3; a high copy number plasmid carrying ampicillin and kanamycin resistance. The replication origin is a pUC19-derived pMB1 (copy number of 100-300 per cell). (http://parts.igem.org/Part:pSB1AK3)

pSB1C3; a high copy number plasmid (RFC [10]) carrying chloramphenicol resistance. The replication origin is a pUC19-derived pMB1 (copy number of 100-300 per cell). (http://parts.igem.org/Part:pSB1C3)

BBa_K197038 (BBb - BseRI/AcuI entry vector); a low copy number plasmid with a ColE1 origin of replication and spectinomycin resistance gene (specR). (http://parts.igem.org/Part:BBa_K197038)

BBa_K157000 (pMA-BBFR); plasmid of unknown copy number carrying ampicillin resistance gene (http://parts.igem.org/Part:BBa_K157000)

None of the inserts in these plasmids encode for toxins or are known to be involved in the production of toxins. Plasmids used are mobilisation defective.

d) Details of genes being modified.

Fluorescent proteins. CYFP, RFP and GFP are widely used as marker proteins and are non-toxic.

Antigen 43 (Ag43) protein To localise proteins on the outer *E. coli* cell wall they will be expressed as a fusion protein with the *E.coli* Antigen 43 (Ag43) protein. Ag 43 is a unique autotransporter that promotes bacterial cell-to-cell aggregation and is encoded by the *flu* gene. Ag 43 can be expressed on the *E.coli* cell surface in large quantities, up to 50000 copies per cell. AG 43 has an N-terminal signal peptide; an N-proximal passenger domain (α domain) that is secreted, an autochaperone domain that facilitates folding of the passenger domain; and a C-terminal β-barrel domain (β domain) that forms an integral outer membrane protein. The passenger domain(α domain) confers the autoaggregation phenotype and it is bound to the surface via non-covalent interaction with the β domain.
The *flu* gene will be modified for the insertion of additional sequences around base position 552 (amino acid position 148) by site-directed mutagenesis. It has been previously shown that this insertions in this region allow for presentation of these sequences on the outer cell wall (doi:10.1128/JB.184.15.4197-4204.2002.). To establish proof of principle we will insert at this position a FLAG epitope tag (or another non-toxic well established peptide tag recognised by commercially available antibodies) flanked by restriction sites.

Ice Nucleation Protein :The *Pseudomonas syringae* Ice Nucleation Protein encoded by gene *inaK.* The protein promotes ice formation responsible for the surface frost damage in plants and has no known toxic effects. Protein sequences fused to INP (as outlined above) have no now toxic effects and we do not expect the combination of

		sequences to be toxic either. Biotinylation Acceptor Peptide/BirA: The Biotinylation Acceptor Peptide is a 15 amino acid long peptide (GLNDIFEAQKIEWHE) where the central lysine is specifically biotinylated by the prokaryotic enzyme biotin holenzyme synthetase, encoded in the BirA gene of *E. coli*. The peptide will be amplified from a iGEM standard part, and expressed as INP or Ag43 fusion protein at the cell surface. Exposure of the peptide on the surface is not harmful or dangerous to the environment. To control for the specificity of the Biotin-streptavidin mediated cell-cell interaction it may be necessary produce an *E. coli* strain lacking the *birA* gene. The birA gene is involved in the control of biotin synthesis and deletion of this gene is not expected to confer new harmful properties to *E. coli*. Epitope tags/Antibody fragments. Initial experiments will involve the exposure of an epitiope tag (FLAG tag or similar) on the *E. coli* cell surface using Ag43 or Ice nucleation protein. The inclusion of an epitope tag will allow testing for interaction with other proteins in *in vitro* test systems as for example antibody-coated sepharose beadsThe inclusion of antibody fragments (if the project reaches this point) will be a further proof-of-principle experiment using well characterised single chain antibodies that will be interacting with protein-coated solid phase. The precise nature of the antibody will be decided on in consultation with Prof Andy Porter, an expert in the use of these antibodies. Expression of epitope tag or antibody fusion proteins is not expected to confer new detrimental properties to *E. coli*, and will not enable it to interact with human cells. All genes except for GFP, which will be expressed under the control of AHL, are constitutively expressed. As the genes are not toxic to humans this does not constitute a risk. **(e) an estimation of culture volumes which will be used** Culture volumes will normally range between 2-5 ml and are unlikely to exceed 500 ml.
10.	**How might the GMM be a hazard to human health?** **Evaluate the severity of the harmful effects if they were to occur.** *Consider* *(a) hazards associated with the recipient organism including ACDP hazard group and the effects of any stable disabling mutations,* *(b) hazards arising directly from the inserted gene,* *(c) hazards arising from the alteration of existing pathogenic traits,* *(d) likelihood and effects of natural gene transfer to other organisms.* *If there are considered to be no harmful effects or only effects of low severity, explain how this conclusion has been reached.*	***a)*** **Hazards associated with recipient organism** *E. coli* K12 and its derivatives are multiply disabled and are designated as Class 1 organisms. We will preferably DH5α, Top10 or possibly other closely related strains with similar genetic background. BL21[DE3] and its derivatives may also be used if high protein expression levels are required. This strain is essentially equivalent to K12. (Chart et al., (2000) J.Applied Microbiol. 89, 1048-1058.) Good microbiological practice will be followed when using these organisms and over many years of use, no adverse effects have been noted. ***b)*** **Hazards arising from inserted gene** As the genes expressed have no known toxic effects, there is minimal risk arising from gene transfer to other bacteria. Deletion of the *birA* gene is not expected to produce a toxic effect and does therefore not present a risk. ***c)*** **Hazards arising from the alteration of existing pathogenic traits.**

		No pathogenic traits are being altered so there is no hazard related to this. ***d)* Likelihood and effects of natural gene transfer to other organisms** Should transfer occur, the nature of most of the genes being manipulated (housekeeping or reporter genes) means deleterious consequences are unlikely in the extreme to result. Plasmids used are mobilisation defective. None of the genes that are to be propagated or expressed in *E.coli* are known to have deleterious effects on human health. It is highly unlikely that even if large amounts of GMM were ingested that the protein would be targeted in sufficient quantity to a location likely to cause detrimental effects.
11.	**Which containment level is necessary to protect human health?** *See HSE guidance on GMMs for requirements of containment levels 1, 2, 3 and 4* *Give details of any additional precautions which are necessary in addition to those of the assigned containment level*	Containment level 1
12.	**Is the required level of containment available in the laboratories and other facilities that will be used for the work?**	Yes
13.	**How might the GMM be a hazard to the environment?** **Evaluate the severity of the harmful effects if they were to occur.** *If there are considered to be no harmful effects or only effects of low severity, explain how this conclusion has been reached.*	Except for BL21 derivatives, the strains used are multiply-disabled and therefore pose no risk to the environment. BL21 is a *E. coli* B derivative and, as proteins expressed are non-toxic, it poses no risk to the environment. In fact BL21 is disabled to an extent it is considered essentially equivalent to K12. (Chart et al., (2000) J.Applied Microbiol. 89, 1048-1058.)
14.	**Are any additional containment measures required to protect the environment in addition to those necessary to protect human health?** *Give details*	No
15.	**Assign the work to an activity class** *Class 1, 2, 3 or 4* *The activity class is equivalent to the containment level except that if some additional precautions from a higher containment level are used, the work must be assigned to the activity class equivalent to that higher level.*	Class 1
16.	***For work provisionally assigned at activity class 2***	

	or above **What factors must be taken into account with respect to health surveillance of people working on this project?** *Provide details of (a) factors that increase the susceptibility of an individual to infection by the genetically modified micro-organism(s), and (b) symptoms of an infection by the genetically modified micro-organism(s).*	
17.	***For work previously approved by the Committee which is now being submitted for 3 yearly review*** **Complete declaration regarding changes** *Tick as appropriate*	There have been no changes to this application since the last review : _____ All changes to this application since the last review have been highlighted: _____

Note1: If the work is assigned to activity class 1, the GMM must present no or negligible risk either to humans or to the environment

Note2: Work assigned to activity classes 2 and above must be notified to the Health and Safety Executive after approval by a Genetic Modification Safety Committee and before work can begin. A notification fee will be payable.

ADDITIONAL INFORMATION

All GMMs in contaminated material and waste must be inactivated by "validated means", the method of inactivation chosen being appropriate to the level of risk.

1.	Will it be necessary for gloves to be worn to protect the laboratory workers from the GMM?	No
2.	Will a microbiological safety cabinet be required to protect laboratory workers from the GMM?	No
3.	Explain how GMMs in contaminated material and waste will be inactivated.	Autoclave (Prestige table-top autoclaves or IMS central autoclave facility)
4.	Explain how the means of inactivation will be validated	Autoclaves are regularly serviced and undergo annual testing to ensure correct operation.
5.	What "degree of kill" is the means of inactivation expected to achieve? How has it been arrived at?	100%. Reasonable expectation of kill of microorganisms exposed to 126°C for 14 minutes (standard autoclave cycle, Prestige Autoclaves). While this autoclave cycle is non-standard (usual cycle is 121°C, 15 min.), our laboratory has experimentally tested the effectiveness of the Prestige cycle, and found it to completely kill *E.coli* cells, in standard 100 ml volumes in flasks placed in the middle of a full autoclave load
6.	If autoclave facilities are to be used, where	IMS 1:21

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	are they located?	
7.	If chemical means of inactivation are to be used, what chemicals will be used and at what concentrations?	1 % Virkon (small volume spills) Virkon powder (large volume spills). 70% ethanol. Chemical means will only be used to disinfect surfaces and in the case of accidental spillage. Decontamination methods are specified in detail in our Local Rules for GM work.
8.	What will be the means of disposal of the inactivated waste? *At Foresterhill liquid waste, after inactivation, will normally be disposed of to drain. Solid waste, after inactivation will normally be sent off site as part of the "orange bag" waste stream. The waste will be macerated to make it unrecognisable and further heat treated before being placed in landfill. Provide details of any alternative or additional means of disposal which will be used.*	Microbiological waste will be disposed of by CFA processing (solid waste) or discarded (autoclaved liquid waste).
9.	What disinfectant will be available for immediate use in event of a spillage? Please specify type and concentration.	Virkon; Powder will be used in liquid spillage situations involving larger volume.
10.	What disinfectant will be used to clean bench tops and laboratory equipment after use? Please specify type and concentration.	Ethanol; 70% v/v

Work must not commence until the proposer has received written approval from an authorised representative of the Foresterhill Genetic Modification Safety Committee.

APPLICANTS ARE STRONGLY ENCOURAGED TO ATTEND THE COMMITTEE MEETING AT WHICH THEIR APPLICATION IS CONSIDERED. FAILURE TO DO SO MAY DELAY APPROVAL AND PREVENT THE PROJECT STARTING.

Signature of Proposer:…… …. Date:……13/06/14……

e-mail: ……b.mueller@abdn.ac.uk…… Telephone:……437536

Submit the completed form to:

Jennifer Craig, PA to HoS & IMS Co-Director, School of Medical Sciences, IMS, Foresterhill – j.craig@abdn.ac.uk

Advice and assistance with genetic modification safety matters can be obtained from
Dr P Cash School of Medicine and Dentistry
Prof J M Collinson School of Medical Sciences
Dr J Crockett School of Medicine and Dentistry

Comments of Genetic Modification Safety Committee

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Date considered
Signature of Biological Safety Adviser

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Part name	Plasmid backbone	Additional information
Ag43 protein		
BBa_K542009	pSB1C3	pLacI Regulated AG43
BBa_K759001	pSB1C3	Aggregation Module inducible by arabinose in E.coli.
BBa_K197001	BBa_K197038	
BBa_K346007	pSB1C3	Antigen 43
Ice nucleation proteins		
BBa_K523013	pSB1C3	Plac + INP-EYFP
BBa_K1111014	pSB1C3	Ice Nucleation Protein fused to Streptavidin BBa_K283010
BBa_K1111012	pSB1C3	Ice Nucleation Protein fused to Streptavidin Alive
BBa_K1111013	pSB1C3	Ice Nucleation Protein fused to Streptavidin Dead
Quorum sensing		
BBa_F1610	pSB1AK3	3OC6HSL Sender Device
BBa_F2621	pSB1A2	3OC6HSL Receiver Device
BBa_K300010	pSB1C3	PoPS-based self-inducible device
BBa_K300029	pSB1C3	3OC6HSL sender device with J23116
BBa_K300024	pSB1C3	Measurement system for K300017 self-inducible device
BBa_K1090000	pSB1C3	AHL signal sender with RFP reporter under lac promoter control
BBa_K1022100	pSB1C3	pBAD:AIP receiver:GFP:TT
BBa_I13272	pSB1C3	YFP Producer Controlled by 3OC6HSL Receiver Device
Antibody/BirA		
BBa_K157003	BBa_K157000	Anti-NIP singlechain Fv-Fragment
BBa_K404246	pSB1C3	The Biotinylation Acceptor peptide motif